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An Adherent Cell Perifusion Technique to Study the Overall and Sequential Response of Rat Alveolar Macrophages to Toxic Substances

by Gilles Forget,* Marie-Josée Lacroix,* Alain Cadieux,* Raymond Calvert,‡ John H. Grose† and Pierre Sirois*

Essentially pure (97%) alveolar macrophages were isolated by bronchoalveolar lavage of rats with warm (37°C) PBS solution. These cells were allowed to adhere to the inside walls of open-ended glass cylinders which were closed off at each end by three-way stopcocks. The adhering cells were perifused with RPMI-1640 medium supplemented with 5% fetal bovine serum for 18 hr at the rate of 1 mL/hr, and the effluent medium was collected automatically in 2-mL aliquots. Cell recoveries and viabilities did not differ from those found for Petri cultures treated similarly, indicating that the perifusion method under study offered an adequate milieu for short-term primary cultures. The alveolar macrophages in culture were subjected to the presence of particulate (chrysotile asbestos) and soluble (phorbol myristate) toxicants, and their response was monitored in the effluent medium by measuring the release of prostaglandins (PGE) by radioimmunoassay. A significant increase in the sequential release of PGE was observed in the presence of asbestos (100 μg/mL) or phorbol myristate (200 ng/mL). Treatment of the cells with indomethacin (20 μM) completely abolished the release of PGE stimulated with phorbol myristate. A cumulative response to the toxicants was also observed when cells were harvested manually from the chambers: asbestos caused a 2-fold increase in cell mortality relative to control, while phorbol myristate brought about a 3-fold increase in the number of dead cells. This effect was not prevented by the presence of indomethacin. Cell aggregation was also observed when cells were perifused in the presence of phorbol myristate, whether indomethacin was present or absent. Our results indicate that the cell perifusion system combines the advantages of conventional adherent cell cultures (viability, aggregation) with those of perifusion techniques (sequential metabolism studies.

Introduction

Alveolar macrophages participate in the initial defense mechanism of the lower respiratory airways to particulate materials (*1-3*). Through phagocytosis, they ingest and remove from alveoli and terminal bronchioli, foreign and endogenous particulate bodies. In this way, they maintain the integrity of the bronchoalveolar milieu. They also take part in the initiation of the inflammatory response to toxic airborne irritants. This they accomplish through the release of several mediators of inflammation, for instance, prostaglandins, lysosomal enzymes and monokines (*4-7*). Until now, conventional cultures of alveolar macrophages have been used to assess the cumulative release of these mediators. More recently, different techniques which permit the culture of various cell types with continuously renewable media have appeared in the literature (*8-13*). These novel methods make possible a sequential analysis of secretion products as the culture progresses. However, most of them do not permit a total recovery of cells at the end of the experiment for the purpose of assessing the viability of the perifused cell population. The method described here combines the advantages of perifusion techniques with those of conventional adherent cell cultures. It has been used to

*Unite de Recherche Pulmonaire, Faculte de Medecine, Centre Hospitalier Universitaire, Universite de Sherbrooke, Sherbrooke, Que., Canada, J1H 5N4.

†Centre de Nephrologie, Hotel-Dieu, Quebec, Que., Canada, G1R 2J6.

‡Department d'Anatomié et Biologie Cellulaire Faculte de Medicine, Centre Hospitalier, Université de Sherbrooke, Sherbrooke, Qué., Canada J1H 5N4.

study the effect of both particulate and soluble cytotoxic compounds on rat alveolar macrophages.

Methods and Materials

Isolation of Rat Alveolar Macrophages

All glassware and plasticware used in these experiments were siliconized and sterilized prior to use. One rat was rendered unconscious by the intraperitoneal administration of 0.8 mL Nembutal (20% Abbott). The abdominal cavity was cut open, and the animal was exsanguinated by sectioning the dorsal aorta and portal vein. The diaphragm was breached to prevent efficient respiratory movements, and the rib cage was cut open along the sternal cartilage. The trachea was denuded and cannulated with a flanged catheter which was tied firmly into place. The other end of the catheter was joined by a three-way stopcock (model K75, Pharmaseal) to a pair of 20-mL syringes. Eight equal volumes (10 mL) of warm (37°C) sterile phosphate-buffered saline (PBS) solution were injected into the lungs by one syringe and aspirated in the other after gentle massage of the lungs essentially as described for the guinea pig by Maxwell et al. (*14*). The lavage fluid was combined and kept on ice until centrifuged at 350*g* for 10 min. Pellets which contained blood, as evidenced by gross discoloration, were discarded, and the lavage-procedure was repeated on another animal. The pellets were resuspended in a combined volume of sterile PBS solution and recentrifuged at 350*g* for 10 min after the determination of cell number and assessment of cell viability by the trypan blue exclusion method. The pellet was resuspended in a volume of RPMI-1640 medium containing *l*-glutamine (Gibco or Flow Laboratories) and an antibiotic/antimycotic solution (penicillin, 1000 U/mL, streptomycin, 1000 μg/mL, and Fungizone, 0.25 μg/mL, Gibco) to yield a final concentration of 10 million cells/mL. Differential cell counts were performed after cytocentrifugation and staining by a combination of Wright and Giemsa stains. Cell pellets containing approximately 10^6 cells were also processed for electron microscopy after fixation in 1% glutaraldehyde and postfixation in osmium tetroxide.

Perifusion of Alveolar Macrophages

The perifusion chamber consisted of an open-ended glass tube (tubing inserts from Corbeil Tissue Culture System, Bellco Glass) measuring 135 mm in length with an inside diameter of 5.5 mm. Each end was occluded by a three-way stopcock (model K75, Pharmaseal) fitted with a piece of Silastic tubing (0.104 in. inside diameter, 0.192 in. outside diameter) over the narrow port to tighten the fit. The efferent end of the chamber was fitted with a piece of nylon filter (Nytex 1 μm pore size, B. & S. H. Thompson) between tube and stopcock (Fig. 1).

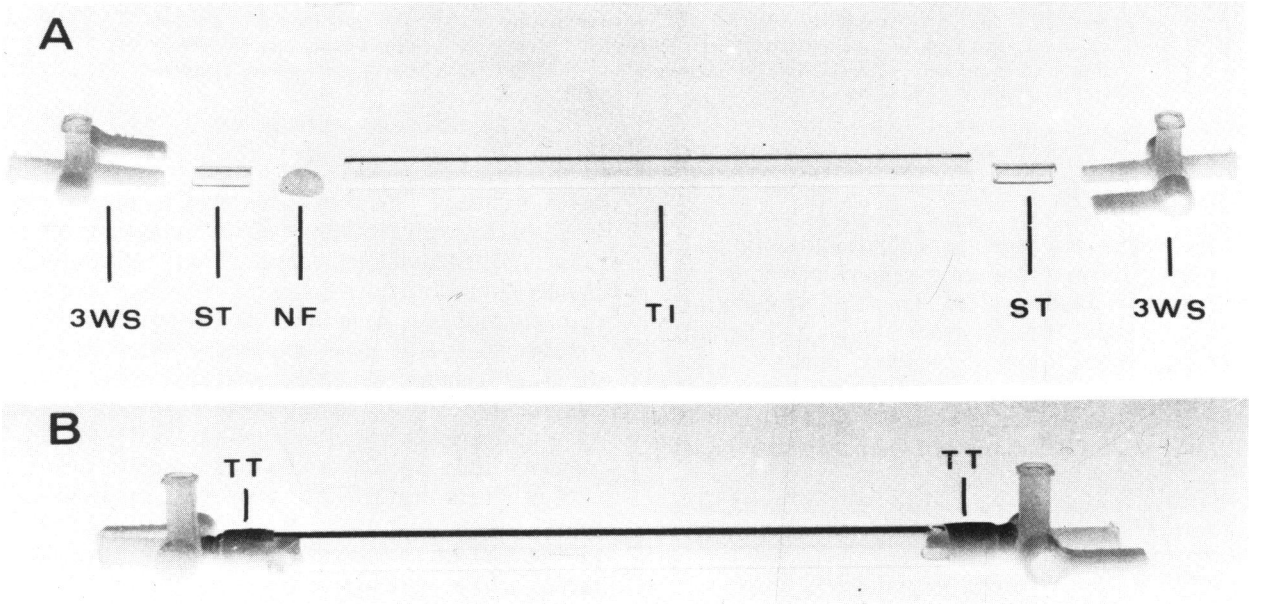

FIGURE 1. Perifusion chamber: (*A*) unassembled chamber showing (3WS) three-way stopcock; (ST) Silastic tubing; (NF) Nytex filter; (TI) tubing insert from Corbeil Tissue Culture system; (B) assembled chamber with (TT) Teflon tape.

Alveolar macrophages (AM) were diluted to 10^6 cells/mL in RPMI-1640 medium containing 20% fetal bovine serum (Gibco or Flow Laboratories), and 3 mL of this suspension were introduced in the perifusion chamber through the afferent stopcock. The chamber inlet and outlet were closed off, and the chambers were incubated for 1 hr at 37°C to allow the adherence of AM to the glass. At the end of the incubation period the fluid was aspirated through the entry port and replaced with an equal volume of fresh RPMI-1640 medium supplemented with 5% fetal bovine serum. The inflow stopcock was connected to a 50 mL syringe containing the same serum-supplemented medium through a sterile Twin Site Venotube 81 cm IV extension with two "Y" injection sites (Abbott). The effluent stopcock was connected to the needle adapter of a venotube extension fitted with a 50 cm length of Intramedic polyethylene tubing (1.7 mm diameter). The assembly was kept at 37°C and perfused with the medium contained in the syringe by a syringe pump (Valey Scientific Co., Model 540 DD) at the rate of 1 mL/hr. The effluent medium was collected automatically in 2 mL aliquots for 18 hr. At the end of the experiment, the cells were harvested by gently scraping the inside walls of the perifusion chambers with a glass rod fitted with a piece of Silastic tubing. The cells were counted, and the viability of the population was evaluated by the trypan blue exclusion method. In a number of experiments, alveolar macrophages were also cultured for 18 hr in a Falcon Petri dish (60 mm diameter) in an incubator so that viabilities and cell retentions could be compared. In certain instances, cells recovered from the chambers were pelleted and processed for electron microscopy.

Canadian chrysotile B asbestos (UICC classification) was sterilized and suspended in RPMI-1640 medium containing 5% fetal bovine serum in a glass Dounce type homogenizer and introduced by the free port of the afferent stopcock in a 3 mL total volume. Soluble compounds to be tested were dissolved in the serum-supplemented (5%) medium contained in the 50 mL reservoir syringe.

Radioimmunoassay of Prostaglandins E

The measurement of prostaglandin E_2 was performed by radioimmunoassay. The final reaction mixture was 470 μL distributed as follows: 300 μL sodium borate buffer (50 *mM*, pH 8.0) containing 0.1% bovine γ-globulin (Sigma), 10 μL (^{3}H) PGE_2 (5 nCi, New England Nuclear), 100 μL sample and 60 μL antibody diluted 1:100 (final dilution 1:780). The reaction was allowed to proceed at 4°C for either 3 or 18 hr. Free radioactive PGE_2 was precipitated out with the addition of 200 μL of dextran-coated charcoal (5 mg Dextran T-70, 250 mg Norit-A in 20 mL borate buffer). After 15 min at 4°C, the preparation was centrifuged in an IEC microfuge (International Centrifuge, Model MB) for 1 min, and 400 μL of the supernatant were mixed with 8 mL of either Bray's scintillation cocktail or Solution-947 (New England Nuclear) for evaluation of the radioactivity by scintillation spectrometry in a LKB-Wallach Rack-Beta scintillation counter. Standards of 10 to 500 pg were used in the standard curve of the assay, and the curve was linear in respect to a logit/log scales graph. All curve fitting and calculations were done on a Radio Shack Model III microcomputer with a program developed in the author's laboratory. The antibody was produced as described previously (*15*). The reactivity of the antibody to other prostaglandins and arachidonic acid metabolites was negligible. The only exception was the high cross reactivity with PGE_1, and for this reason, the results obtained were assigned to PGE activity instead of PGE_2.

Statistics

Results are expressed as the mean ± SEM of a minimum of five observations. The significance of observed differences was tested by the Student's *t*-test for either paired variables or for two means as the case warranted, as stipulated in the text. The results were considered significant at the 0.05 or 0.01 levels of confidence.

Results

Evaluation of the Perifusion Method with Respect to Cell Viability

The mean number of free alveolar cells obtained per rat was $1.52 \pm 0.8 \times 10^7$ cells (mean ± SEM, N = 28). Preparations containing more than 18 million cells were considered atypical and discarded. Viabilities exceeded 85% in all preparations with a mean of 90.1% ± 0.6. Differential cell counts performed after cytocentrifugation indicated a consistently high alveolar macrophage count (97%) in contrast to contaminant cells (lymphocytes, 2%; pneumoctyes, ≤ 1%).

The perifusion culture method was compared to a conventional static Petri culture. The Falcon plastic Petri dish had a bottom surface of 28.3 cm² compared to a total internal surface of 23.3 cm² for the perifusion chamber. However, microscopic observation of the perifusion chamber disclosed that the AM were mainly adhered to the bottom half of the available surface. Notwithstanding this, the perifusion chambers retained by adherence as many cells as the Petri dishes. After 18 hr, there was no significant difference between the number of cells retained by the perifusion chambers and those harvested from the

Petri dishes, indicating that cells did not migrate out of the chamber during perifusion (Table 1). Cell viability was also compared for the two methods and was not found to differ significantly (Table 1).

Chambers containing AM were perifused in the presence of either dialyzed (Gibco) or undialyzed (Flow Laboratories) fetal bovine serum (5%). A statistical evaluation of cell viabilities under those two conditions revealed that the undialyzed serum was significantly more efficient in preserving cell viability than the dialyzed serum (Table 1).

Electron microscopic observation of the cell preparations before and after an 18-hr perifusion were indicative of ultrastructural integrity (Figs. 2 and 3). The surface of the AM obtained immediately after bronchoalveolar lavage is covered with numerous pseudopodia, and this feature is also apparent in cells which have been perifused for 18 hr. The cytoplasm of the fresh cells contains the normal complement of organelles with a prominence of primary and secondary lysosomes. Residual bodies are also common. The Golgi apparatus is well developed, and the cytoplasm contains elongated mitochrondria and large clear vacuoles. A small number of granular endoplasmic reticulum cisternae are present, together with polyribosomes and free ribosomes.

AM which have been perifused for 18 hr appear normal, as they do not differ from the preceding cells. The nucleus is normal, and the cytoplasm contains a very large number of primary and secondary lysosomes, together with many residual bodies. The mitochrondria appear normal in all respects, as does the Golgi complex. There seems to be a larger complement of granular endoplasmic reticulum cisternae, and clear vacuoles are scarce and reduced in size. In general, the electron microscopic appearance of the cells which have been perifused for 18 hr is not significantly different morphologically from freshly obtained cells.

Table 1. Comparison of cell culture parameters and methods

Culture method	Retention (18 hr), mean ± SEM (*N*)[a]	Mortality, % mean ± SEM (*N*)
Plastic Petri dish (60 mm diameter)	$1.24 \pm 0.06 \times 10^6$ cells (7)	16.6 ± 1.5 (6)
Perifusion chamber	$1.17 \pm 0.04 \times 10^6$ cells (6) N.S.[b]	17.1 ± 1.7 (6) N.S.[c]
Perifusion chamber (5% dialyzed serum)		22.9 ± 0.6 (6)
Perifusion chamber (5% undialyzed serum)		14.5 ± 1.2 (24), $p < 0.01$[b]

[a]The original inoculate was 3×10^6 cells in all cases. After 1 hr preincubation, medium and nonadherent cells were removed and new medium introduced.

[b]*t* test for individual means performed on actual cell counts.

[c]*t* test for paired values (performed on the actual cell counts).

Effect of Particulate and Soluble Toxicants on Perifused AM

The sequential liberation of PGE was assessed with respect to soluble and particulate toxicants. Canadian chrysotile B was introduced in a perifusion chamber (100 μg/mL) which has perifused for 18 hr against a control chamber containing serum-supplemented medium only. As illustrated in Figure 4, asbestos stimulated the metabolism of arachidonic acid in rat AM. Prostaglandins E levels, which averaged less than 1 ng/2 hr in untreated perifused cells, increased progressively during the first 6 hr of perifusion in fiber treated cells and maintained a plateau of approximately 1.8 ng/million cells/2 hr of incubation. The increase observed in the presence of asbestos was significant between 10 and 16 hr of perifusion. The cytotoxicity of the asbestos fibers was indicated by a significantly higher mortality in that chamber (44%) against that of the control chamber (21.9%) ($p < 0.01$).

Phorbol myristate is a membrane-active compound which has been shown to stimulate the release of prostaglandins from macrophages *in vitro* (*16*) and was tested in the present system (200 ng/mL). In order to verify specificity of the PGE release, a third chamber containing phorbol myristate and indomethacin (20 μ*M*) was run in parallel to the control and phorbol myristate (Fig. 5). The synthesis of PGE stimulated by this agent increases rapidly and steadily during the first 8 hr, after which it slowly diminishes, although it remains much higher than in control cells throughout the whole period. However, the presence of indomethacin, an inhibitor of cyclooxygenase (*17*), in the perifusion medium, nullifies the phorbol myristate-induced release of PGE in a significant fashion.

The viability of AM is strongly diminished by phorbol myristate both in the presence (33.9%) or in the absence of indomethacin (32.9%) in respect to the control chamber (10.4%) ($p < 0.01$). Phorbol myristate also produced cell aggregation, another effect which was not inhibited by indomethacin. This observation was obtained at the end of the perifusion experiments, when the harvested cells were counted and their viability evaluated. In both cases, the aggregation was marked (79% and 80.1%), while it was very low in the control chambers (9%). These results are tabulated in Table 2.

Electron microscopy of cells perifused in the presence of phorbol myristate (Fig. 6) shows cells which are strikingly different from fresh or control perifused cells. The most striking feature is the modification in a large number of cells of the surface pattern. The thin pseudopodia have been replaced by numerous blebs all around the periphery of the macro-

phage. The nucleus is normal. The cytoplasm has a disorganized appearance; it contains fewer lysosomes and residual bodies. Occasional lipid droplets can be seen and the cristae are abnormal in many mitochrondria.

Discussion

One problem encountered with conventional cell cultures is the ever-present possibility that accumulating metabolites and/or secretory products can in-

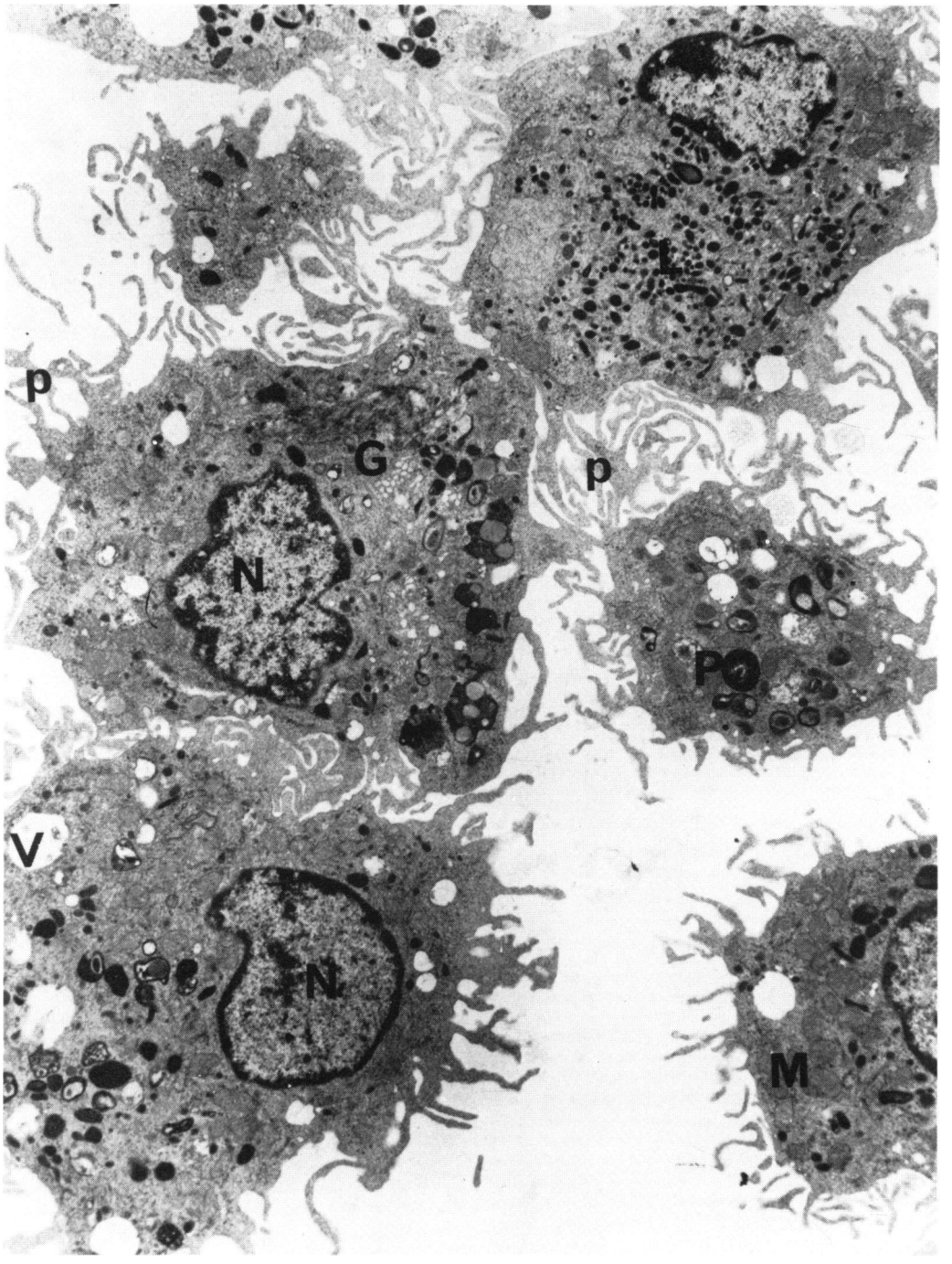

FIGURE 2. Freshly isolated rat alveolar macrophage (original magnification 6703×): (N) nucleus; (L) lysosome; (G) Golgi complex; (R) granular endoplasmic reticulum; (P) phagosome; (p) pseudopod; (M) mitochondrion; (V) vacuoles.

fluence the outcome of the experiment through positive or negative feedback. Several methods have been proposed in recent years to overcome this by maintaining cell cultures in a flow of continuously renewed medium. This has generally been accomplished along two main lines. Either the cells are superfused in a column containing a support (*8*, *18*) or they are perifused in a chamber on filters (*9-13*). The purpose of the present study was to design a cell culture system which combined the advantages of both

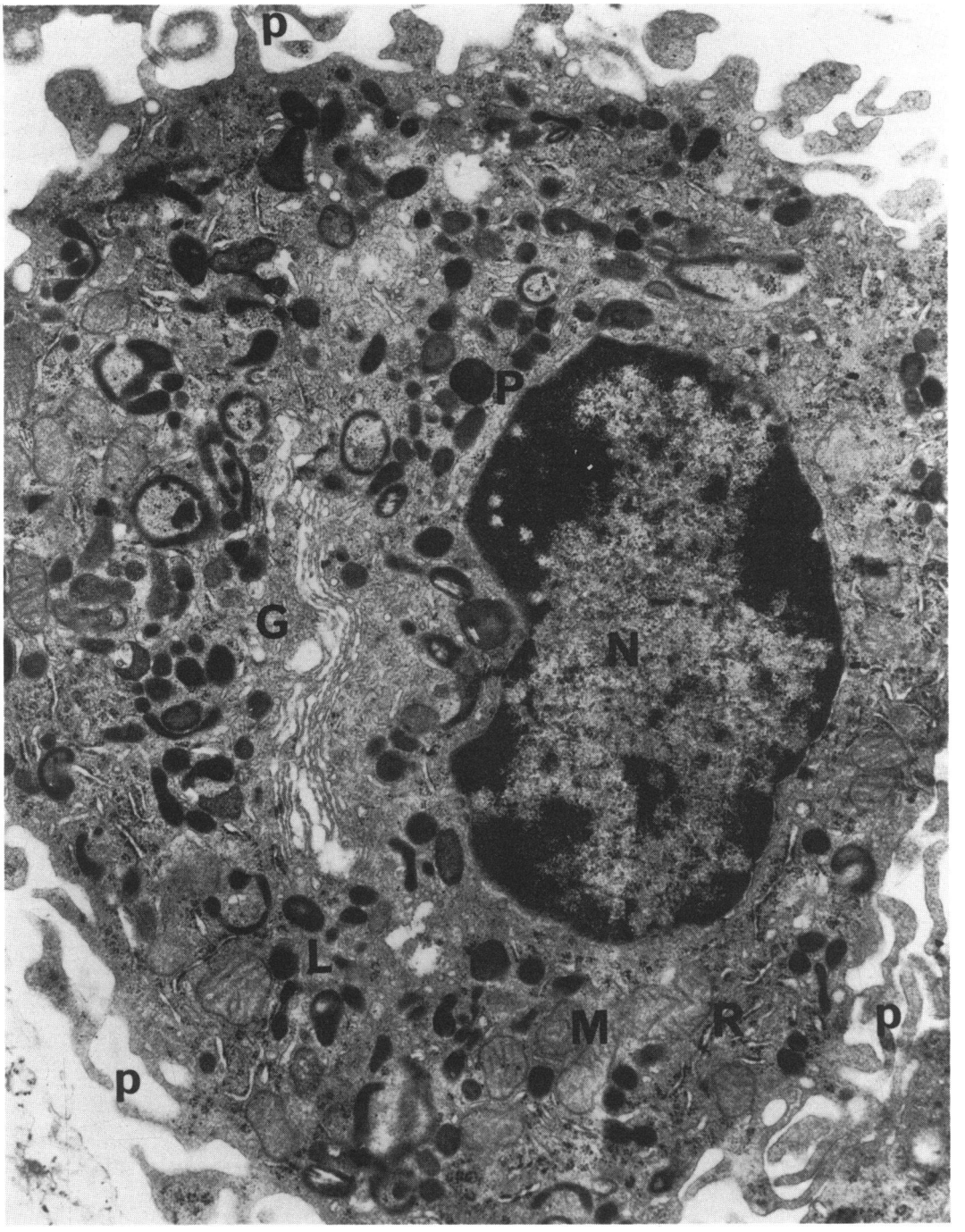

FIGURE 3. Rat alveolar macrophage after 18 hr perifusion in serum supplemented (5%) RPMI-1640 medium (original magnification 17,806 ×): (N) nucleus; (L) lysosome); (G) golgi complex; (R) granular endoplasmic reticulum; (P) phagosome; (p) pseudopod.

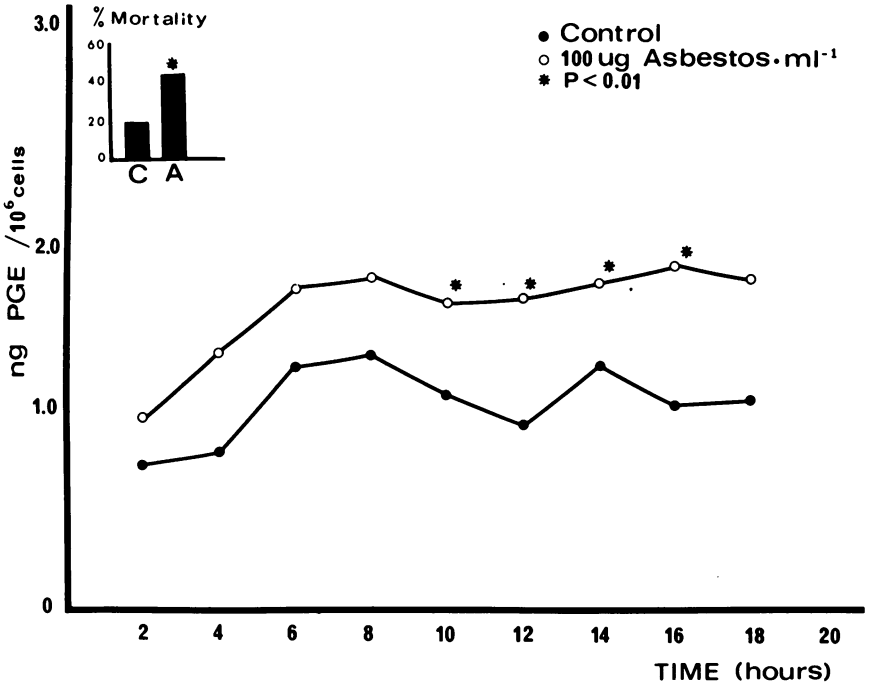

FIGURE 4. Time course of PGE secretion (O) in the absence and (●) in the presence of Canadian chrysotile asbestos. Inset: histogram of cell mortality after 18 hr perifusion.

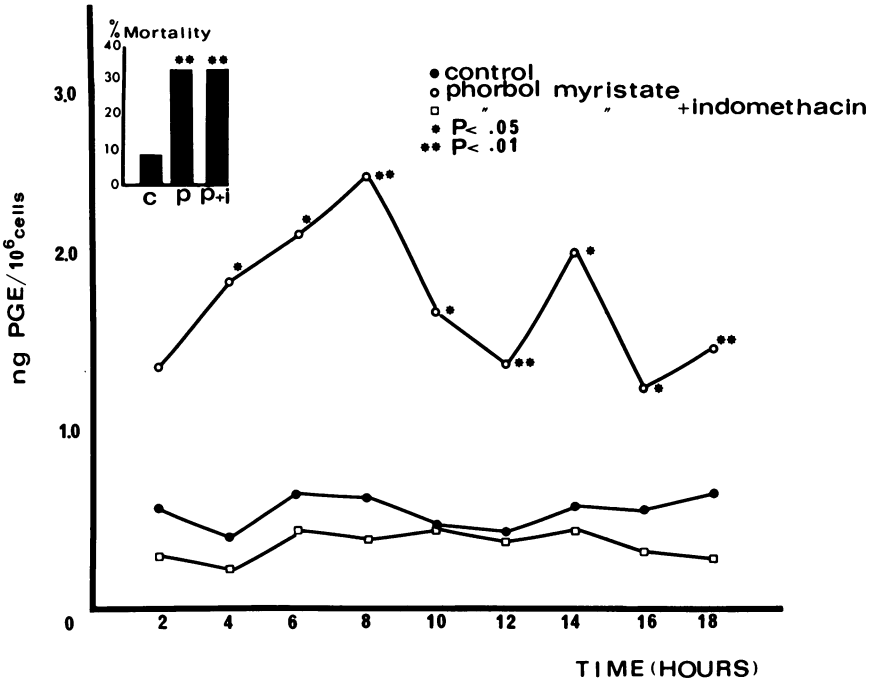

FIGURE 5. Time course of PGE secretion (●) in the absence and in the presence of either (O) phorbol myristate or (□) phorbol myristate and indomethacin. Inset: histogram of cell mortality after 18 hr perifusion.

Table 2. Characteristics of phorbol myristate-induced cell aggregation.

Chamber	Mean number of aggregates ($\times 10^3$) 2-5 cells/aggregate	5-10 cells/aggregate	>10 cells/ aggregate	Free cell[a]	Mean cell total	% aggregated
Control	33	0	0	954	$1.18 \pm 0.03 \times 10^6$	9
Phorbol myristate	55	37	28	252	$1.22 \pm 0.02 \times 10^6$	78.6
Phorbol myristate + indomethacin	49	36	45	223	$1.22 \pm 0.04 \times 10^6$	80.2

[a]Free nonaggregated cell.

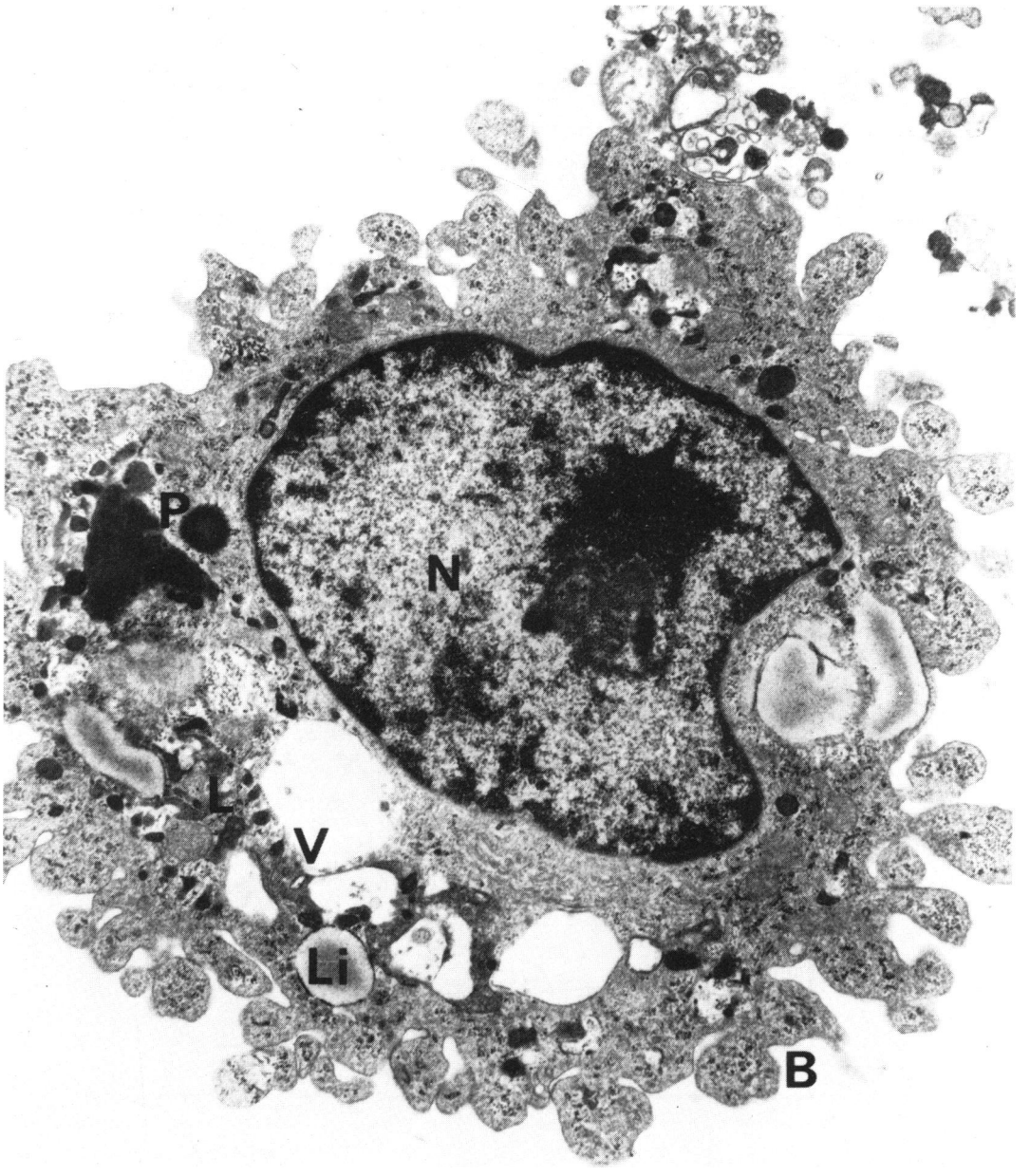

FIGURE 6. Rat alveolar macrophage after 18 hr perifusion in the presence of phorbol myristate (original magnification 11,403 ×): (N) nucleus; (V) vacuoles; (P) phagosome; (Li) lipid droplets; (B) blebs; (L) lysosome.

the traditional primary adherent cell culture with the additional boon of renewed medium and sequential analysis of cell output.

Differential cell counting by cytocentrifugation indicates that the relatively simple lavage method used on the rat lung provides a very homogeneous, virtually pure alveolar macrophage population. Because the medium is renewed between the preincubation of cells in the chamber and the actual perifusion experiment, only the adhering and viable cells are retained in the chamber at the beginning of the experiment. The present technique is the only perifusion culture technique to date which permits the elimination of dead or moribund cells before the perifusion is initiated.

Parallel studies of Petri and perifused cultures indicate that there is no significant migration of AM from the perifusion chamber. This is no doubt facilitated by the filter position at the effluent end of the chamber and by the relatively low flow rate of perifusion. Moreover, the matching viabilities in both Petri and perifused cultures strongly suggest that the perifusion chamber can offer an adequate milieu on which the AM can thrive just as they are wont to do in traditional cultures. The adequacy of the chambers to maintain viable cells in short- and medium-term cultures is further emphasized by the normal aspect of the AM as seen by the electron microscope after 18 hr of perifusion.

The use of dialyzed serum appears to be less beneficial to the AM than the undialyzed counterpart. This is reflected by the increased viability concomitant with the use of undialyzed serum. It is of note that the baseline output of PGE in control chambers is lower and more regular with the undialyzed serum (Fig. 4) than with dialyzed serum (Fig. 3). This points to the fact that the conditions optimal to well-being of the AM are very sensitive to minor modifications.

The level of PGE output cannot be lowered significantly between baseline levels by the addition of indomethacin in the medium, although this potent inhibitor of prostaglandin synthesis (*17*) is capable of preventing phorbol myristate-stimulated release of PGE. While this finding is difficult to explain, the blocking effect of phorbol myristate-mediated release of PGE is unequivocal, and points to the specificity of the observed stimulation. This stimulation has been reported by Chang et al. (*16*) for traditional cultures of murine peritoneal macrophage. However, these authors report a plateau in the release of PGE after 2 hr, while in the present system, the release of PGE is linear during the first 8 hr followed by a slow decline, still above control levels during the rest of the perifusion experiment. These observations could indicate that a negative feedback mechanism, which was at work in the murine macrophage monolayer culture, was circumvented in the perifusion system because of medium replacement.

Asbestos-mediated release of prostaglandins from macrophage has already been described using adhering macrophage cultures (*5*, *7*, *19*, *20*). The present findings confirm these reports. The perifusion system can thus be used to study inflammatory responses of AM to either soluble or particulate toxic substances. Just as important, this technique allows direct observation of the cells following the perifusion to assess such criteria as viability and aggregation. It was thus possible to observe that apart from causing release of PGE, both asbestos and phorbol myristate can cause cell death. The two compounds produced 2- and 3-fold cell mortalities in the perifused populations, respectively. Interestingly enough, the addition of indomethacin to the perifusion medium in the presence of phorbol myristate did not prevent mortality. It could thus indicate that PGE synthesis and liberation is not necessarily related to cell death. The aggregation was not measured in asbestos experiments. The AM were aggregated onto asbestos fibers, and these prevented precise easy evaluation of the size of aggregates.

The technique presented in this study has permitted the evaluation of several important criteria of inflammation and cytotoxicity. It was valuable both with particulate and soluble compounds. Comparison with traditional methods of cell cultures indicates that the perifusion system is just as valid for the preservation of cell viability. It can prove invaluable in the sequential study of many processes and still allow the evaluation of criteria which were until now not easily quantifiable in the course of the available perifusion methods.

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